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Antitumor activity and pharmacokinetics of podophyllotoxin incorporated into solid lipid nanoparticles

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To evaluate the antitumor activity and pharmacokinetics of podophyllotoxin (PPT) incorporated into solid lipid nanoparticles (SLN), Kunming mice inoculated with flesh tumor were used as animal model. The mice received a single daily intraperitoneal injection of PPT in 20% ethanol (5 mg/kg) and PPT-SLN (5 mg/kg in PPT) for 3 weeks. Gross tumor volumes, body weight and clinical observations were recorded daily. The mice were sacrificed for 24 h after the last administration, and the tumor inhibition rate was calculated with the tumor weight. For the pharmacokinetics research, the mice were treated with intraperitoneal injection of PPT (10 mg/kg) and PPT-SLN (10 mg/kg in PPT). Blood samples were collected at different time to determine the PPT concentration in plasma by HPLC. Blood drug level-time curve was made and pharmacokinetic parameters were calculated. As a result of drug administration, the tumor volume and weight of the mice injected with PPT-SLN were significantly restrained compared with mice treated with PPT or negative control. The tumor inhibition rate of 58.13% showed a significant antitumor activity of PPT-SLN. At the same time, the increased weight gain of the mice injected with PPT-SLN suggested a reduced toxicity of PPT in SLN. Pharmacokinetics study displayed a higher blood concentration, a prolonged circulation time, and an increased bioavailability of PPT-SLN compared with those of PPT. Our results demonstrated that PPT-SLN could optimize pharmacokinetics, enhance antitumor activity and attenuate toxicity, so it has a promising prospect for the application in anti-tumor treatment.

podophyllotoxin, solid lipid nanoparticles, tumor

1 Introduction

Podophyllotoxin (PPT) is a naturally occurring lignan extracted from *Podophyllum* (Berberidaceae). The explosion of research applications of PPT to cancer therapy was triggered by Kaplan's application of podophyllin to venereal warts in 1942[1]. Scientists proved the antineoplastic effect of PPT in 1947[2], which induced extensive research on it. However, utility of PPT as an antitumor drug encountered a lot of restrictions because of its toxicity[3]. Recently, efforts are made to develop new derivatives or dosage forms of PPT with optimized curative effect and attenuated toxicity[4,5]. In previous researches, we carried out deep investigations about the antitumor mechanism of PPT and its derivatives[6–8].

In recent years, nanomaterials have been paid much attention to for its unique characters[9–14]. Among these various nanoparticles, solid lipid nanoparticles (SLN), which can absorb or package drugs in its liposome core, are a kind of innovative nano-liposomes drug carrier containing high-melting point liposomes (e.g. triglycerides, and stearic acid)[15]. The favorable biocompatibility,

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low toxicity and delayed drug release demonstrate the suitability of the SLN as a promising drug delivery system for PPT.

We have successfully synthesized the PPT-loaded SLN (PPT-SLN). As expected, SLN could significantly reduce the toxicity of PPT and improve its ability of tumor cell suppression *in vitro*. Moreover, it is found that PPT-SLN exhibits desired stability, average diameter and encapsulation efficiency[16]. In this study, further work was carried out to investigate the tumor inhibitory effect and pharmacokinetics of PPT-SLN *in vivo*.

2 Experimental

2.1 Materials

PPT was kindly provided by the Center of Analysis and Measurement, University of Science and Technology of China; PPT-SLN was prepared by a procedure reported elsewhere [16], and was dissolved in normal saline before use. All reagents and solvents were of analytical or HPLC grade and were used without further purification. For the quantitative determination of PPT, a high-performance liquid chromatography (HPLC) was used (Agilent 1100 system).

2.2 Mice

Female Kunming mice weighing 18—22 g were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. and housed in stainless steel cages in a ventilated animal room. Room temperature was maintained at 24±2℃, relative humidity was 50±10%, and light cycle and dark cycle alternate every 12 h. Distilled water and sterilized food for mice were available *adlibitum*.

2.3 *In vivo* studies in tumor-bearing mice

2.3.1 Animal tumor model and intraperitoneal administration. Mice Sarcoma 180 (S180) cells were obtained as a gift from Shanghai Cancer Institute and maintained *in vivo* in Kunming mice in an ascites form. S180 cells were collected from ascites, and were adjusted to a concentration of 1×10^7 cells/mL in normal saline. For tumor implantation, 0.2 mL of S180 cell suspension was injected subcutaneously into mice in the right flank. In 24 h after tumor inoculation, mice were randomly sorted into treatment groups with each having 12 mice and received a single daily intraperitoneal injection for 3 weeks, of saline (negative control), PPT in 20% ethanol (5 mg/kg), PPT-SLN (equimolar to PPT in the ethanol formulation), and cyclophosphamide (CTX, 20 mg/kg, positive control).

2.3.2 Antitumor effects. Mouse body weight and tumor volume were monitored daily. Tumor volume was calculated based on the equation $(a \times b^2)/2$ (a: length, b: width). Mice were sacrificed in 24 h after the last administration. Tumor was separated and weighed to determine the tumor inhibition rate, which is calculated according to the formula: inhibition rate (%) = (1–tumor weight in test group/tumor weight in control) × 100%. The statistical significance of the data was evaluated by the Student's t test. $p < 0.05$ was considered significant.

2.4 Pharmacokinetics research

2.4.1 Administration of PPT-SLN and PPT to mice. PPT-SLN and PPT formulations were injected intraperitoneally at the PPT dose of 10 mg/kg body weight, and each group consisted of five animals. Blood samples were collected in heparin-containing tubes at the designated time (10, 20, 40 min and 1, 2, 4, 8, 16, 32, 48 and 60 h) via quickly removing the eyeball from the socket with a pair of tissue forceps. Plasma was isolated by centrifugation (10 min at 5000 r/min), and stored at –20℃.

2.4.2 Plasma sample treatment. 1 mL plasma was mixed with 0.4 mL ethyl acetate. After vortex for 10 min and centrifuging for 10 min (13000 r/min), the organic phase was separated and evaporated, and the residue was then reconstituted with 200 μL mobile phase and was mixed in a vortex mixer for 10 min. After centrifugation at 13000 r/min for 10min, a portion (20 μL) of the reconstituted sample was injected onto the chromatography column.

2.4.3 HPLC determination of PPT. PPT concentration was analyzed by HPLC using a Discoery C_{18} column (250 mm×4.6 mm, 5 μm particle size) and an isocratic program with a solvent system of methanol/water 45∶55 (v/v) with a UV detector at 290 nm (flow rate 1 mL/min).

2.4.4 Statistics and pharmacokinetics analysis. Pharmacokinetic parameters, including area under the curve (*AUC*), total body clearance (*Cl*) and plasma half-life for the distribution and elimination phase ($t_{1/2}\alpha$, $t_{1/2}\beta$) were assessed using a software program (3P87).

3 Results and discussion

3.1 Therapeutic efficacy of PPT-SLN in tumor-bearing mice

Mean tumor volumes are shown in Figure 1. Rapid

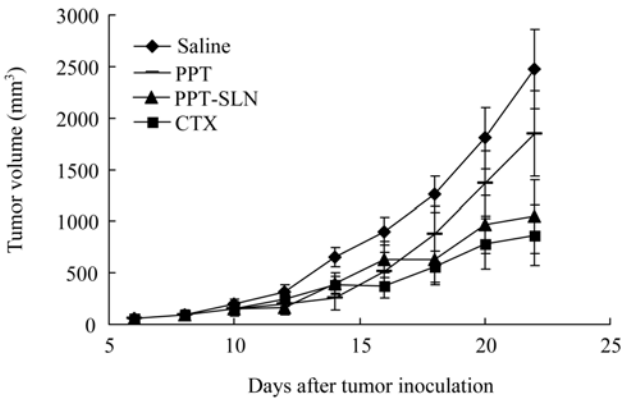

Figure 1 *In vivo* antitumor effect by i.p. injection of PPT and PPT-SLN at a dose of 5 mg PPT/kg in S180 tumor-bearing Kunming mice evaluated by solid tumor growth. Mice were inoculated s.c. in the right flank in 24 h before the first administration of drugs. The mice were received a single daily intraperitoneal injection for 3 weeks. The control was given the same volume of saline or CTX. Points, mean; bars, SE.

tumor growth was observed in negative control mice. PPT-SLN exhibited a higher anti-tumor effect compared with PPT or saline, probably due to their long circulation. Thus, PPT-SLN was demonstrated to be more effective than free PPT in inhibiting tumor growth. Tumor inhibition rates were calculated and are shown in Table 1. In the same way, mice injected with PPT-SLN displayed higher inhibition rate than that of free PPT. Body weights of the mice were monitored throughout the experiment as an indication of adverse effects of the drug. As shown in Table 1, loss of body weight in mice injected with PPT-SLN was less than CTX and free PPT, which proved the better biocompatibility of PPT- SLN.

3.2 Pharmacokinetics studies

3.2.1 Chromatographic performance. Figure 2 shows HPLC chromatograms for extracts from standard PPT, plasma spiked with PPT, and plasma sample from mice treated with PPT. No interference from serum constituents was observed for either assay system.

3.2.2 The calibration curve of PPT in serum samples. The linearity of the method was conducted using drug-free plasma with PPT added to yield concentrations of 0.1, 0.5, 1.0, 2.0, 5.0, 10.0, and 50.0 μg/mL. Data were obtained through linear regression analysis of peak height ratios of PPT (*A*) versus PPT concentrations

Table 1 Antitumor effect of different drug formulations reflected by body weight, tumor mass, and tumor inhibition rate

Drug formulation	Body weight at different times (g)		Tumor mass (g)	Tumor inhibition rate (%)
	initial drug delivery	last drug delivery		
Saline	18.43±1.13	29.68±2.47	2.03±0.31	
PPT-SLN	17.78±1.17	27.89±2.32	0.85±0.19 [a)]	58.13
PPT	18.82±1.28	21.74±2.08	1.24±0.24 [b)]	38.90
CTX	19.44±1.05	20.31±1.89	0.74±0.20 [a)]	63.65

Statistical significances are: a) $p < 0.01$, and b) $p < 0.05$.

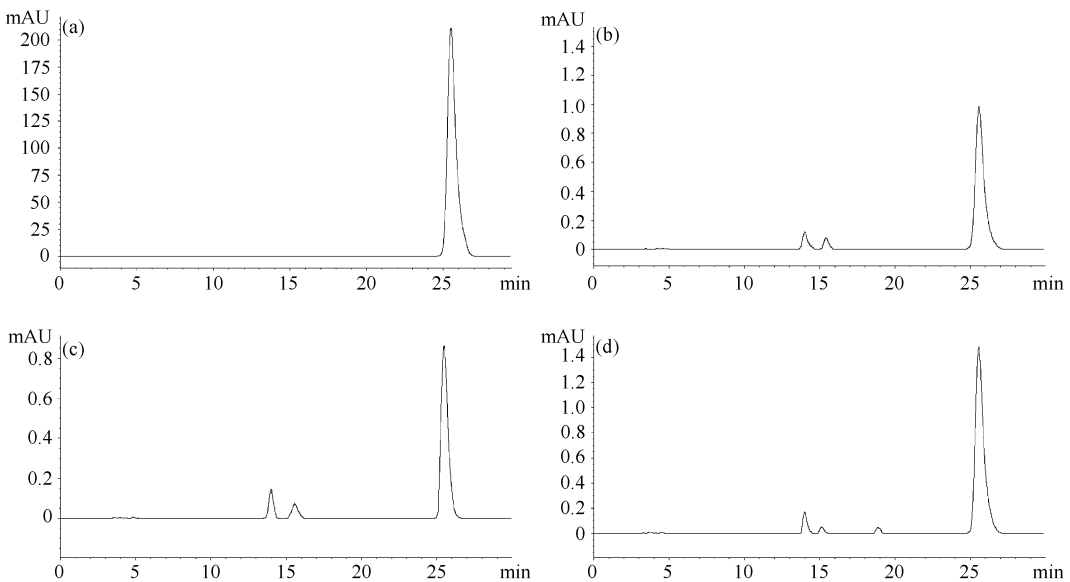

Figure 2 Representative chromatographs of (a) standard PPT; (b) plasma spiked with PPT; (c) plasma sample from the mice added with PPT; (d) plasma sample from the mice added with PPT-SLN.

(μg/mL) in spiked plasma samples (*C*). A typical calibration curve presented the regression equation of $A=4.618C-8.243$ ($r=0.9997$, $n=5$). The calibration curve for the determination of PPT in plasma was linear over the range of 0.1－50 μg/mL.

3.2.3 Precision and recovery of PPT added to serum samples. The within-day and between-day precision was evaluated at three concentration levels (0.1, 10.0, and 50.0 μg/mL) based on five measurements carried out in a single day and over five days of validation period, respectively. The absolute recovery of the extraction was determined by comparing the peak area obtained from the plasma sample with peak areas obtained by the direct injection of PPT standard solutions in the mobile phase at three different concentration levels. The within-day and between-day precision was less than 15%, and the absolute recovery of PPT from the plasma was above 80%.

3.2.4 Pharmacokinetics properties. The plasma PPT concentration-time curves are illustrated in Figure 3. At all times, PPT plasma concentrations were higher in PPT-SLN-treated group than PPT-treated group. The curve meets the two-compartment model according to 3p87 program, and the pharmacokinetic parameters for PPT-SLN and PPT injection are listed in Table 2.

4 Conclusion

The research on nano-drug delivery system has become the hot spot in recent years. It was reported that the antitumor activity of PPT, when mixed with nano- liposome, could be significantly enhanced[17]. The SLN we have synthesized is a novel drug delivery system different from nano-liposome. It has been proved that SLN could depress side effect, improve drug solubility and achieve delayed release, which brings forth application prospect for drugs with poor physiochemical properties[1–20]. PPT is such an anticancer drug limited by the poor physiochemical properties. We have formerly confirmed the favorable solubility for PPT-SLN, thus presenting the possibility for its *in vivo* delivery.

In this study, cyclophosphamide (CTX), a frequently used anticancer drug, was adopted as the positive control. The results of the efficacy studies with murine S180 tumor showed that the tumor growth exhibits a significant difference between positive control and negative control ($p < 0.01$), indicating the reliable data and reasonable error. The remarkable therapeutic effect of PPT-SLN was demonstrated by the statistical significance of tumor weight and volume between mice injected with PPT-SLN and saline ($p < 0.01$). While the free PPT, when administered at the molar equivalent dose, was less effective than PPT-SLN. Results from this murine tumor study also showed the inhibition of weight gain in mice injected with PPT or CTX. In addition, high dose of PPT was even found to kill mice in our preliminary experiment (data not shown). However, PPT-SLN is less toxic, viewed from the body weight increasing in mice administered with PPT-SLN, which represents a reduced side effect of PPT.

Plasma clearance profiles of PPT-SLN were compared with those of free PPT at the molar equivalent dose. PPT-SLN displayed a longer systemic circulation time relative to free PPT, suggesting its greater *in vivo* stability. Serum concentration-time data of mice administered with PPT and PPT-SLN were calculated using

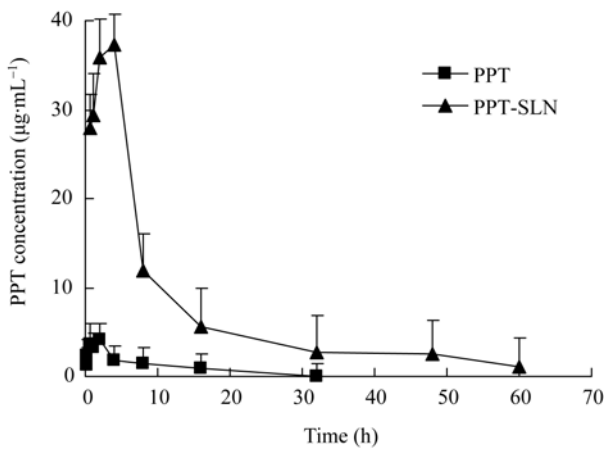

Figure 3 Plasma concentration vs. time curves for PPT and PPT-SLN in mice. PPT and PPT-SLN formulations were administered via i.p. injection at a dose of 10 mg/kg in PPT. Each data point is the average of three to five animals and error bar equals one standard deviation.

Table 2 Pharmacokinetic parameters

	AUC ($\mu g \cdot mL^{-1} \cdot min$)	*CL* ($mg \cdot kg^{-1} \cdot h^{-1} \cdot \mu g \cdot mL^{-1}$)	T_{max} (h)	C_{max} ($\mu g \cdot mL^{-1}$)	$t_{1/2}\alpha$ (h)	$t_{1/2}\beta$ (h)
PPT	63.58±8.48	0.16±0.02	1.23±0.09	3.87±0.67	0.88±0.06	29.93±4.72
PPT-SLN	426.22±60.29	0.023±0.003	2.07±0.35	38.98±5.83	1.54±0.49	20.63±4.08

3p87 software. The results showed that both groups exhibit two-compartment model, which have an initial redistribution phase with a short half-life ($t_{1/2}\alpha$) followed by an elimination phase with a longer half-life ($t_{1/2}\beta$). Encapsulation of PPT in SLN obtained marked differences in terms of the pharmacokinetic parameters. Using SLN as carriers, a seven-fold enhancement of *AUC* was acquired, with an eight-fold reduction of *CL*, which suggests the increase of PPT entering body, and the decrease of PPT eliminated by organism, respectively. Furthermore, animals administered with PPT-SLN exhibit an increased peak serum concentration and a delayed time to peak concentration of PPT compared with those treated with free PPT. Conceivably, this sustained PPT release mechanism is favorable to the suppression of tumor and may account for the *in vivo* efficacy experiment. In conclusion, there is a potential for a broad range of new therapeutic applications using SLN as drug carriers.

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